Organophosphorous Pesticide Exposure Increases the Frequency of Sperm Sex Null Aneuploidy

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It has been estimated that 4 of 1,000 live births and 35% of spontaneous abortions are aneuploid and that an important proportion of embryo and newborn aneuploidy is of paternal origin. Exposure to organophosphorous pesticides (OP) has been associated with sperm hyperploidy/polyploidy. Therefore, we aimed to assess the frequency of sperm aneuploidy (X, Y, and 18) and its relationship with urinary OP metabolites in agricultural workers. We performed multicolor fluorescence *in situ* hybridization on samples from nine men obtained before and during the pesticide spraying season to assess sperm aneuploidy. We measured urinary OP metabolite levels by gas-liquid chromatography. Aneuploidies were found in 0.67% of total sperm nuclei. The most frequent aneuploidy was the lack of a sexual chromosome or sex null (0.19%), followed by XY18 (0.15%) and XY18-18 (0.06%). OP metabolites detected at higher concentrations were dimethylthiophosphate, dimethyldithiophosphate, and diethylphosphate (DEP). There were no differences in average aneuploidy frequency or urinary metabolite levels between samples collected before and after exposure. However, Poisson regression analysis adjusted for age, alcohol intake, and sperm concentration showed significant associations between OP metabolite concentrations and increased frequency of sperm aneuploidies. The association was more evident between DEP and sex null, and the risk increased further during the spraying season. Thus, OP exposure could interfere with sperm chromosome segregation and increase the risk for genetic syndromes, such as Turner's. Further studies are required to assess the prevalence of spontaneous abortions, birth defects, and genetic syndromes in agricultural communities. *Key words:* organophosphorous pesticides, sex null, sperm aneuploidy, Turner syndrome. *Environ Health Perspect* 109:1237–1240 (2001). [Online 24 November 2001]
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In recent years much attention has focused on the potential for a wide range of xenobiotics to interact with and disrupt human reproductive health and genetic homeostasis (*1–3*). Chemical exposures may alter reproductive behavior and contribute to subfecundity, infertility, pregnancy loss, growth retardation, intrauterine fetal demise, birth defects, and testicular and ovarian failure. An abnormal number of sex and autosomal chromosomes in male and female germ cells may cause pregnancy loss, pregnancy delay, spontaneous abortion, and fetal and prenatal mortality or may be responsible for chromosomal/malformation syndromes in newborns (*1,2*). The majority of aneuploidy is believed to be due to nondisjunction during meiosis (*4*). It has been estimated that 4 of 1,000 live births and 35% of spontaneous abortions are aneuploid and that an important proportion of aneuploidy occurring in embryos and newborns are of paternal origin (*5,6*). Paternal contribution to these aneuploidies range from 10% to 100%, depending on the specific chromosome involved (*7*). It has been reported in a series of 66 cases of sex chromosome monosomy that 53 (80.3%) had maternal X, indicating that paternal sex chromosome

loss is the most common error leading to this condition (*6*).

In humans, age, lifestyle (alcohol intake and smoking), and chemotherapy can increase risks for sex-chromosome and autosomal aneuploidies (*5,8–13*). Exposure to organophosphorous pesticides (OP), used worldwide, has also been associated with hyperploidy/polyploidy in sperm (*14,15*) Aneuploidy is a common cause of poor reproductive outcomes in humans and is associated with severe medical problems in live newborns presenting genetic syndromes. In addition, chromosomes X and Y are more frequently damaged than autosomal chromosomes (*16*). On the other hand, it has been postulated that chemicals inducing crosslinks increase the probability of chromosomal nondisjunction (*7,17*). Therefore, OP could induce sperm aneuploidies by interfering with chromosome migration during meiosis because they alkylate DNA (*16,18*). Therefore, we aimed to assess the frequency of sperm aneuploidy for chromosomes X, Y, and 18 by means of three-color chromosome sperm–fluorescence *in situ* hybridization (FISH), and its relationship with urinary OP metabolites in men before and during pesticide spray season.

Methods

Study population. The study was conducted in the agricultural community of Villa Juarez, State of Durango, Mexico. We chose nine healthy men (mean age 32.4 years, range 18–47) with no history of chemotherapy, radiotherapy, or chronic illness from a subset of subjects participating in a longitudinal study on reproductive effects of OP exposure. Four individuals were pesticide sprayers and the rest were agricultural workers who worked on the field but were not directly involved in spraying pesticides. Participants have been residents of this community for more than 15 years. The community is surrounded by agricultural fields whose main products are vegetables. Methyl parathion, metamidophos, endosulfan, and dimethoate were the pesticides applied most frequently. An informed consent signed form was obtained from each participant. The study was approved by the Ethics Committee of the School of Medicine, University of Coahuila, Mexico.

Semen collection and analysis. Two semen samples were collected from each participant after at least three days of sexual abstinence. Specimens were collected into clean plastic containers at a provisional andrology laboratory installed in the agricultural community. The first sample was collected during crop preparation when small quantities of pesticides are sprayed in January and February (henceforth called "before OP spraying season"). The second

was collected in May and June 1998 at the beginning of the heavy spraying season when large quantities of pesticides are sprayed (henceforth called "during OP spraying season"). After liquefaction, samples were analyzed according to World Health Organization criteria (*19*). Aliquots of whole semen were frozen at –70°C until they were transported on dry ice to the University of California, Los Angeles, for FISH analysis. Samples were thawed and sperm concentration was adjusted to 100 million per milliliter; thereafter, eight air-dried smears were prepared from each sample.

Fluorescence in situ *hybridization.* These procedures have been adapted from Robbins et al. (*5*). We used a commercial kit (CEP X spectrum orange, Y spectrum green, and 18 spectrum aqua direct-labeled fluorescent DNA probes; Vysis, Inc., Downers Grove, IL, USA). The kit detects alpha satellite sequences in the centromere region (Xp 11.1–q11.1) of chromosome X, in the satellite III DNA at the Yq12 region of chromosome Y, and at the 18p region (18p 11.1–q11.1) for chromosome 18. We used an axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY, USA) to score slides as described by Robbins et al. (*5*). The signal for chromosome X was detected with green fluorescence, the Y with red, and the chromosome 18 with aqua fluorescence. We defined aneuploidy as an abnormality of the chromosome number. Disomy was considered when two fluorescent domains of the same color occurred within the sperm head,

comparable in brightness and size and at least one domain apart. We recorded 12 chromosome patterns (X18, Y18, XX18, YY18, X18-18, Y18-18, 0-18-18, 0-18, XX18-18, YY18-18, XY18-18, and a category for other patterns). Total aneuploidies included all the abnormal chromosome patterns found in this study. All slides were coded before analysis and scored in random order. Approximately 10,000 (range 9,944–10,250) sperm nuclei per slide were scored for each semen sample. Scorers were blinded to subject identity and sperm collection time. Each slide was scored by two scorers and splits compared for variability in scoring. There were no significant differences between scorers (variability < 10%). Only cells consistent with sperm size and shape or with a visible tail were scored.

Pesticide analysis. A spot morning urine sample was collected from each participant before semen sample collection and frozen (–70°C) until analysis. We measured OP metabolites dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), and diethylthiophosphate (DETP) at CINVESTAV according to Aprea et al. (*20*). We calculated total dialkylphosphates (DAP) as the sum of the five metabolites. Organochlorine (OC) pesticide serum levels were determined by gas-liquid chromatography according to U.S. EPA (*21*).

Statistical analysis. We tested differences in average aneuploidy frequency or urinary metabolite levels for samples collected before and during spraying seasons using the

Mann-Whitney *U*-test. Descriptive statistics were expressed as geometric means. We tested the association between aneuploidy frequency and urinary OP metabolites with Poisson regression using a generalized estimating equation to account for the lack of independence of observations and the skewed distribution of semen aneuploidy (*22*). Because urinary concentration ranges for each metabolite were very different, the coefficients (β) calculated with data from all subjects were multiplied by the interquartile range (75–25) of each metabolite and exponentiated to express associations in terms of relative risk of aneuploidy (*22*). We analyzed the relationships between sex null aneuploidy and urinary OP metabolites before and during the pesticide-spraying season using Poisson regression. The regression coefficient for urinary OP obtained in these models can be interpreted as the logarithm of the ratio of expected aneuploidies per unit of increase in urinary OP metabolites with all other explanatory variables held constant in the multivariate models. We assessed age, alcohol intake, smoking, sperm concentration, and DDE serum levels as confounders in multivariate models. All analyses were performed using the statistical software STATA 6.0 (Stata Corp., College Station, TX).

Results

Median semen parameters for the group as a whole were as follows: abstinence period 3 days, range 2–4; total concentration 62.5 million, range 16–170 million; sperm concentration/mL 43 million, range 19–85 million; total motility 80%, range 20–90%; viability 81%, range 20–91%. We observed no significant differences between exposure seasons.

The analysis of the total sperm nuclei evaluated for the three chromosomes studied (180,972) in the whole group of individuals showed an abnormal chromosome constitution in 0.67% of sperm (Table 1) with a per subject range of 0.42–0.99%. The ratio of Y- and X-bearing sperm was 1:0.97. The

Table 1. Grouped aneuploidy data for study participants before and during pesticide spray season (per 10,000 cells).

Chromosome constitution	Before (*n* = 9) 90,382 cells	During (*n* = 9) 90,590 cells	Total (*n* = 18) 180,972 cells
Sex null	18.97 (6–27)	19.95 (10–48)	19.45 (15–25)
XY18	14.23 (9–25)	15.71 (6–29)	14.96 (12–19)
XY18-18	6.02 (1–14)	7.71 (4–14)	6.82 (5–10)
YY18	2.18 (1–3)	5.32 (4–7)	3.32 (2–5)
YY18-18	2.91 (2–5)	3.45 (2–6)	3.19 (2–4)
XX18-18	2.07 (1–3)	3.87 (2–7)	2.78 (2–4)
Y18-18	2.44 (1–4)	2.60 (2–4)	2.51 (2–3)
X18-18	2.02 (1–4)	1.96 (1–4)	1.99 (1–3)
XX18	2.00 (1–3)	2.59 (1–6)	2.24 (2–3)
018-18	1.00 (—)	0 (—)	1.00 (—)
Other	2.11 (1–4)	4.07 (2–7)	3.00 (2–4)
Total aneuploides	59.25 (42–82)	72.44* (50–99)	65.51 (25–74)

*p = 0.07, Mann-Whitney test.

Table 2. Urinary OP metabolite (OPm) levels before and during the pesticide spray season.

OPm (ng/mL)	Before (*n* = 9)	During (*n* = 9)
DMP	0.04 (0.0025–3.24)	0.04 (0.0025–2.78)
DEP	1.63 (0.038–138.7)	0.81 (0.038–118.1)
DMTP	39.9 (0.89–1346.8)	120.04 (1.05–7151.9)
DMDTP	6.25 (0.4–224.9)	8.00 (0.61 –182.8)
DETP	0.14 (0.04–0.73)	0.11 (0.04–0.55)
Total DAP	97.74 (3.8–1538.9)	157.84 (6.3–7355.9)

Results are shown as geometric mean (range).

overall hybridization efficiency was 99%. The most frequent aneuploidy was the lack of a sexual chromosome or sex null (0.19%), followed by XY18 (0.15%) and XY18-18 (0.06). Diploidy was found in 262 sperm nuclei (0.146%) and the most frequently observed was XY18-18. There were no significant differences in the prevalence of the three most frequent aneuploidies when compared before and during the spraying season. However, the frequency of total aneuploidies was slightly higher during the spraying season (Table 1). The small number of subjects precluded a meaningful assessment of the influence of occupation on the frequency of abnormal sperm chromosome constitutions. We found no significant differences or associations when analyzing 10 other nonfrequent chromosome patterns (Table 1). There were no significant differences between urinary DAP levels before or during the pesticide spraying season. In both seasons, the metabolite detected at highest concentration was DMTP, followed by DMDTP and DEP (Table 2). DDE was the only OC found in serum samples.

Poisson regression analysis using a generalized estimating equation showed significant associations between DEP (β = 0.00022; p = 0.0001) and sex null. Smaller but still significant associations were also observed with DMTP and total DAP. The frequency of total aneuploidies was associated with DMDTP (β = 0.00009; p = 0.0001). Smaller associations were also observed with DMTP and total DAP (Table 3). The relative risk for sex null associated with the interquartile range of DEP (1,381 ppb) was 1.36 (95% confidence interval 1.18–1.55), whereas DMTP and total DAP presented smaller but still statistically significant relative risks. Similarly, we observed smaller risk increases for total aneuploidies with DMTP, DMDTP, and total DAP. Furthermore, the stratified analysis by spraying season showed higher slopes for DETP (β = 0.0043; p = 0.0001) and DEP (β = 0.00053; p = 0.0001) and higher relative risks for DEP (2.59) and DETP (1.68) for sex null during the spraying season (Table 4).

Discussion

Our most important finding was the direct association between the increased frequency of sex null sperm aneuploidy and OP metabolite levels. To our knowledge, this is the first study reporting increased sex null frequency in a population exposed to OP in an agricultural setting. The frequency was outside the range recently reported in nonsmoking healthy men ([7]). Sperm sex aneuploidies may be responsible for some of the most common genetic syndromes, such as Turner (46,XO) and Klinefelter (46,XXY). Recent evidence has shown that Turner syndrome has an estimated frequency of 1–2% among all clinically recognized pregnancies and that 70–80% of 46,XO patients retain the maternal X chromosome, because the paternal X chromosome is missing ([23]). Our findings were in agreement with the increased frequency of total sperm aneuploidies (0.30%) found in workers of a Chinese factory manufacturing methyl parathion, ethyl parathion, and metamidophos, compared with that of their controls (0.19%) ([15]). A moderately increased prevalence of chromosomes X, Y, and 18 sperm aneuploidies was also observed in these workers but nullisomy was not scored ([15]). Methodologic differences between laboratories and scorers preclude meaningful comparisons of absolute percents of sperm sex null aneuploidies among studies, because it is possible that differences between the Mexican and Chinese studies may have a technical component in addition to potential

differences in exposure. In contrast, a recent study on Danish farmers showed no significant effects on the frequency of aneuploidy (disomy or diploidy) of sperm chromosomes 1 and 7 before and after their seasonal dithiocarbamate fungicide exposure ([24]). The frequency of disomy XY18 and diploidy XY18-18 recorded for the whole group in our study is within the range reported in healthy men ([9,25–27]).

Regarding possible mechanisms for these aneuploidies, it has been postulated that chemicals inducing crosslinks (DNA:DNA and/or DNA:protein) increase the probability of chromosomal nondisjunction, possibly through disturbances in recombination or kinetochore and microtubule perturbations during cell division ([7,17]). Therefore, OP could induce sperm aneuploidies by interfering with chromosome migration during meiosis I and II, because they alkylate DNA and form adducts that may in turn be converted to interstrand crosslinks ([16,18]). In our study, overall hybridization efficiency was 99%. Therefore, it is unlikely that our results stemmed from poor sex chromosome hybridization efficiency. In addition, the second and the third main aneuploidies found in our study included both sex chromosomes (XY-18 and XY-18,18). If poor sex chromosome hybridization were the cause for the increased frequency of sex null, other aneuploidies should have been found and not those including both sex chromosomes (XY-18 and XY-18,18). Sex nulls are a product of altered meiosis I or meiosis II; however, the

Table 4. Relationships between sex null aneuploidy frequency and urinary organophosphorous pesticide metabolites (OPm) before and during the pesticide-spraying season.

OPm	Before spraying season ($n = 9$)			During spraying season ($n = 9$)		
	β^a	RR	95% IC	β^a	RR	95% IC
DMP	−0.04	0.81	0.91–1.01	0.007	1.01	0.98–1.03
DEP	0.13*	1.28	1.01–1.43	0.53**	2.59	1.59–2.71
DMTP	0.02	1.09	0.63–2.18	0.002	1.02	0.99–1.04
DMDTP	−0.95*	0.74	0.38–1.00	0.10	1.07	0.99–1.11
DETP	−2.20	0.89	0.60–1.00	4.30*	1.68	1.13–2.81
Total DAP	−0.01	0.90	0.47–1.58	0.003	1.05	0.99–1.08

Results were calculated using Poisson regression. RRs were adjusted for age, alcohol intake, and total sperm concentration. The interquartile ranges (75–25) used to calculate RRs before the spraying season were DMP: 4,926; DEP: 1,936; DMTP: 3,661; DMDTP: 307; DETP: 50; and Total DAP: 8,043 (ppb). During the spraying season were: DMP: 1,865; DEP: 1,801; DMTP: 10,079; DMDTP: 702; DETP: 122; and Total DAP: 19,204 (ppb).
[a]β was multiplied by 1,000. *$p < 0.05$. **$p < 0.001$.

Table 3. Relationships between sperm aneuploidy frequencies and urinary OP metabolites (OPm).

OPm	Sex null ($n = 18$)			XY18 ($n = 18$)			XY18-18 ($n = 18$)			Total aneuploidies ($n = 18$)		
	β^a	RR	95% CI	β^a	RR	95% CI	β^a	RR	95% CI	β^a	RR	95% CI
DMP	−0.006	0.99	0.97–1.02	0.017	1.02	1.00–1.04	0.001	1.00	0.07–1.03	0.004	1.00	1.00–1.01
DEP	0.22**	1.36	1.18–1.55	−0.11*	0.86	0.73–1.00	−0.14	0.82	0.66–1.02	0.012	1.02	0.95–1.09
DMTP	0.003*	1.02	1.01–1.04	−0.004	0.97	0.93–1.00	0.006	1.04	0.99–1.08	0.002	1.02	1.01–1.03
DMDTP	0.10	1.05	0.99–1.11	−0.20*	0.90	0.82–1.00	−0.24*	0.88	0.78–1.00	0.09	1.05	1.02–1.08
DETP	−0.052	0.99	0.83–1.18	0.15	1.02	0.85–1.22	0.78	1.11	0.82–1.5	−0.68	0.91	0.83–1.00
Total DAP	0.003*	1.04	1.01–1.08	−0.003	0.96	0.91–1.01	−0.005	0.94	0.87–1.01	0.003	1.04	1.02–0.05

Abbreviations: CI, confidence interval; RR, relative risk. Results were calculated from a generalized estimating equation with Poisson link. RRs were adjusted for age, alcohol intake, and total sperm concentration. The interquartile ranges (75–25) used to calculate RRs were DMP: 969.2; DEP: 1381.1; DMTP: 6239.4; DMDTP:513.1; DETP: 134.3; and Total DAP: 11821.4 (ppb).
[a]β was multiplied by 1,000. *$p < 0.05$. **$p < 0.001$.

lack of an effect on XY sperm suggest that the effect may derive from meiosis II.

OP exposure can be assessed adequately in humans by determining dialkylphosphates in urine. This measurement integrates exposure from sources ranging from direct occupational exposure to indirect dietary exposure (*28*). However, the rapid metabolism and excretion of OP and their metabolites hinders adequate exposure assessment. In our study, the subjects were intermittently exposed to OP at variable concentrations because spraying occurred every 2–3 weeks during the spraying season and it was difficult to define the precise moment and magnitude of exposure. On the other hand, Robbins et al. (*5*) have shown for other genotoxic agents that only the cells in the meiotic window of the spermatogenesis continuum were sensitive to the induction of aneuploidy. In our study, the peculiarities of both exposure and effect precluded a better estimation of the exposure–response relationships. Finally, the relatively small sample size of nine individuals caused large variation that could influence the power to detect smaller differences in exposures and other effects.

In conclusion, we have shown in this preliminary work a positive association between OP metabolite levels and sex null and total aneuploidy frequencies even after controlling for age and lifestyle, factors playing important roles in aneuploidy induction (*5,8–10*). Although DMTP was the metabolite excreted in higher proportion, DEP and DETP showed the strongest association with sex null, suggesting the need for further studies regarding their potential sex null aneuploidogenic properties. Further studies are also required to assess the prevalence of genetic syndromes, such as Turner, subfecundity, spontaneous abortions, and birth defects in exposed agricultural communities.

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